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ARTICLE

Immune response profiling in patients with traumatic injuries associated with alcohol ingestion

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Abstract

Traumatic injuries afflict more than 5 million people globally every year. Current and past animal research has demonstrated association among alcohol, trauma, and impaired immune function, whereas human registries have shown association between alcohol and morbidity as well as mortality. The purpose of this study is to elucidate the immune interactions with alcohol in traumatically injured patients. We prospectively enrolled 379 patients after trauma at three medical centers in the Surgical Critical Care Initiative. Plasma was analyzed using Luminex for up to 35 different cytokines. Collected samples were grouped by patients with detectable plasma alcohol levels versus those without. Univariate testing determined differences in analytes between groups. We built Bayesian belief networks with multiple minimum descriptive lengths to compare the two groups. All 379 patient samples were analyzed. Two hundred eighty-two (74.4%) patients were men, and 143 (37.7%) were White. Patients had a median intensive care unit length of stay (LOS) of 5.8 days and hospital LOS of 12 days. Using single variate analyses, eight different cytokines were differentially associated with alcohol. Cytokines IL-12 and IL-6 were important nodes in both models and IL-10 was a prominent node in the non-alcohol model. This study found select immune function differed between traumatically injured patients with measurable serum alcohol levels as compared with those without. Traumatically injured patients with positive blood alcohol content appear less able to inhibit inflammatory stress. Alcohol appears to suppress pro-inflammatory IL-12 and IL-6, whereas patients without alcohol have greater levels of anti-inflammatory IL-10 expressed at injury and may better regulate anti-inflammatory pathways. Future studies should determine the relationship with these markers with clinically oriented outcomes.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Alcohol intoxication alters the immune response to trauma, resulting in higher mortality rates. This traumatic stress immune response has been studied in retrospective

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population-based studies and animal models but not at the individual level in human subjects with traumatic injury.

WHAT QUESTION DID THIS STUDY ADDRESS?

We sought to bridge the existing evidence that links alcohol to impaired immune function in traumatically injured patients. We hypothesized that there would be distinct cytokine differences between traumatically injured patients with blood alcohol content versus those without alcohol in their blood at the time of injury.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Select immune function measures differ between traumatically injured patients with measurable alcohol levels compared to those without. Alcohol appears to suppress pathways involving IL-12 and IL-6. Patients without alcohol have greater levels of IL-10.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Serial testing of specific immune cytokines could help predict clinical course and allow earlier intervention to prevent infection or suppress dysregulated immune response.

INTRODUCTION

Globally, traumatic injuries afflict more than 5 million people annually and cause harm to millions more. It is estimated that trauma accounts for ~ 10% of global mortality and is a global threat. For every death, hundreds of thousands of emergency department visits and hospitalizations contribute to significant long-term morbidity, resulting in a large proportion of patients with temporary or permanent disability.[1] Alcohol is often a contributing factor in traumatic injuries and the incidence of trauma in individuals intoxicated with alcohol appears to be escalating.[2] Studies have shown that 40% of victims of traumatic injury have positive blood alcohol concentrations and 35% of those presenting with trauma have blood alcohol levels above the legal limit of intoxication (> 0.05%).[1]

There is consensus that acute alcohol intoxication alters the immune response to systemic stress induced by trauma, resulting in higher mortality rates in patients consuming alcohol compared with sober patients.[3] Yet, to date, the potential cellular level alterations in this traumatic stress immune response in humans has only been studied in retrospective population-based studies or animal models.[4,5] Studies show that alcohol affects the immune response in cell lines in a multifaceted manner, including inhibition of both inflammatory cytokine production (IL-4, IL-6, IL-10, IL-11, and IL-13) and activation of neutrophils.[6] Homeostatic neuroendocrine counter-regulatory pathways are also markedly disrupted by alcohol intoxication and lead to both blood flow compromise and increased injury to end organs.[2] Recent literature suggests that alcohol exposure in the uninjured healthy individual causes disruption of cytokine release and recruitment of immune cells to surround or clear the immune stimulus.[7] The resultant cytokine signaling dysregulation that occurs from alcohol consumption often then contributes to immune-related pathologies.[8] Fully identifying the complex mechanisms in which alcohol affects these traumatically injured subjects, however, has remained elusive. In fact, some studies have shown that low to moderate blood-alcohol content (BAC) may be beneficial in patients with severe brain injury from blunt head trauma.[9]

Our overarching goal is to bridge the existing evidence from animal-based research and registry-based human research linking alcohol and immune function in the setting of trauma. The purpose of this study is to validate previous findings regarding the interaction between immune function and alcohol in the setting of traumatic injury and to identify specific elements of immune response differences between patients who enter the emergency department with both a traumatic injury and a positive BAC versus those without evidence of BAC. We hypothesized that distinct cytokine differences would occur between traumatically injured patients with a positive BAC versus those with a negative BAC at the time of injury. Understanding these differences can assist clinicians in better treating traumatically injured patients with a positive BAC and can increase overall understanding of the impact of alcohol on the immune system.

METHODS

We prospectively enrolled patients requiring inpatient admission for a traumatic injury to one of three medical centers that participated in the SC2i.[10] Participating clinical facilities included Walter Reed National Military Medical Center (Bethesda, MD), Duke University Medical Center (Durham,

NC), Emory University Hospital (Atlanta, GA), and Grady Memorial Hospital (Atlanta, GA). We enrolled both military and civilian patients suffering traumatic injuries that included motor vehicle collisions (31.8%), stab wounds (9%), gunshot wounds (46.4%), falls (2.4%), and other traumatic injuries. Exclusion criteria included patients over the age of 80 years, prisoners, self-inflicted injuries, or those from whom we were unable to obtain consent during the first week of their inpatient admission for any reason. We collected clinical data from patients and from chart reviews. Samples from trauma patients were collected as soon as clinically feasible following their injury with the mean being 1 day of hospitalization. All patients' serum alcohol levels were measured as part of usual care upon presentation. We collected whole blood and isolated serum at the sites for the SC2i Consortium Biorepository following consortium-wide standard operating procedures and Good Clinical Laboratory Practice and stored until use for these analyses. The patient or an appropriate healthcare proxy provided informed consent. The study was approved by our respective institutions' institutional review boards prior to any patient data collection. The methods of sample collection and preparation have been previously reported but are briefly encapsulated here for clarity (Supplementary Material).[11]

Sample preparation

Frozen serum samples were thawed on wet ice for filtering. The samples we ran had two freeze-thaw cycles. We do not believe this impacted the stability of the analytes tested.[12] We mixed them and transferred 400 µl to a 1.5 ml tube. Samples were centrifuged at 12,000 revolutions per minute (RPM) for 15 min at 4°C and a 350 µl sample was transferred into a 0.65 µm filter tube (Millipore, Billerica, MA). They were centrifuged at 12,000 RPM (17,709 G) for 30 min at 4°C. We then aliquoted 120 µl of the filtered samples into clean tubes, flash froze the samples in liquid nitrogen, and stored them in a −80°C freezer.

After filtering, we thawed samples and used a 1:50 dilution of samples. We made a standard cytokine preparation per kit instructions. A 1× wash preparation was created and used for all washes. Standard plates were prepared and we added 50 µl of sample, standards, and incubation buffer to all wells and allowed to incubate for 2 h. After washing, we added detection antibodies and incubated samples for 1 h. After another wash, streptavidin-RPE was added and samples were incubated for 30 min. We washed these again and resuspended beads in wash buffer. For the 32-plex analysis, we used a Human Cytokine 30-plex panel kit supplemented with a custom Human 2-plex panel (Invitrogen; Cat. No. LH6003 and LCP0002) for 32 cytokines (IL-1α, IL-1β, IL-1ra, IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, GM-CSF, G-CSF, INF-γ, INF-α, TNF-α, EGF, bFGF, HGF, VEGF, Eotaxin, MCP-1, MIP-1α, MIP-1β, RANTES, MIG, and IP-10). For 35-plex analysis, we used Invitrogen Catalog #LHC6005M kits are from Thermo Fisher Scientific. The Comprehensive Human Cytokine Magnetic 35-Plex Panel provides reagents for the accurate, reproducible, and sensitive quantitation of human proteins including EGF, Eotaxin, FGF-basic, G-CSF, GM-CSF, HGF, IFN-alpha, IFN-gamma, IL-1 beta, IL-1 alpha, IL-1RA, IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40/p70) IL-13, IL-15, IL-17A, IL-17F, IL-22, IP-10, MCP-1, MIG, MIP-1 alpha, MIP-1 beta, RANTES, TNF-alpha, and VEGF.

Data analysis

The overarching goal of this project was to understand the systemic differences between trauma patients with a positive BAC and trauma patients with a nonpositive BAC (negative BAC). By building system models of different patient groups and comparing them to each other to determine how the cytokine and chemokine interactions differ, our goal was to further elucidate the immune function level interactions occurring in patients suffering trauma in those with measurable serum alcohol content compared with those without.

In pursuit of this goal, we built Bayesian belief networks (BBNs), using FasterAnalytics[13] version 7.0 (DecisionQ Information Operations, Inc., Arlington, VA). A BBN starts with an observation, then calculates the relationships of various variables that are likely to impact that observation based on known probabilities (beliefs). BBNs are very useful for illustrating the differences in the immune systems of different patient groups because they are represented by acyclic directed graphs. These graphs can be overlaid on each other so that analysts can easily observe where there are differences in the cytokine and chemokine joint probability distributions. In this case, BBNs can identify which relationships exist between cytokines, allow measurement of the magnitude of relationships and the number of relationships between cytokines for any given variable, handle a large number of variables without large datasets, and compare which relationships exist and how many relationships exist between the two groups of subjects (positive BAC vs. negative BAC). These models also easily deal with missing data, which was anticipated in this cytokine data set.

For each kit (32-plex, 35-plex, and combined), we built four BBNs for preliminary modeling using minimum descriptive lengths (MDLs) of 0.01, 0.05, 0.10, and 0.15. For each MDL, we built one model on the full data set and 10 models on 10 training sets, each containing 90% of the data. For each MDL, we looked at 11 models and found which variables were first-degree associates of any measurable serum alcohol on arrival. We used these variables in final modeling. If a

variable in either kit was missing for more than 20% of the records, we dropped the variable for the models with that kit.

Power analysis

To determine the effect size of our sample, we used the R function pwr.t2n.test from the pwr CRAN package. For the purposes of this project, we ran the power analysis six times.[14] In one test, we used the 2 sample sizes available in the data, a power level of 0.8, and a significance level of 0.05. We also ran two more calculations with the same power and significance level settings but adding in the effect size and leaving out one of the sample sizes. We ran one calculation with an effect size of 0.5 and one with an effect size of 0.2.

Model derivation and validation

We split each MDL data set into two data sets, one with patients who tested positive for alcohol and one with patients who tested negative. We split each data set randomly into 10 training and 10 test sets, and we built 10 cross-fold models along with the model on the full data set. To validate the models, we used a similarity scoring tool wherein all variables in the data set are predicted and given a score of 0 or 1, depending on whether the model was able to correctly predict what variables were using the other variables. The scores were averaged to give the record one score between 0 and 1, indicating how well the model predicted the variables. We considered records with a score closer to 1 ($\geq$0.5) to be similar to the data the model was built on (e.g., either patients with alcohol in their blood or patients without alcohol in their blood). We considered patients with a score closer to 0 (<0.5) to be dissimilar to the data that was used to build the model. We tested each record on both the alcohol and nonalcohol models, so records were given two scores: how close they were to the alcohol model and how close they were to the nonalcohol model. We used these scores to build receiver-operating characteristics (ROC) that measured how well the models differentiated between alcohol and nonalcohol patients at various cutoffs.

We used 10-fold cross validation. The scores from each set of 10 test sets were used to build an ROC and measure the area under the curve (AUC). We considered any AUC of 0.6 or above to be a good differentiator between the two cohorts. The images of the alcohol and nonalcohol models were overlaid so that we could see the differences in the populations. We also provided statistics about the connectivity of the models. Mann–Whitney *U* Tests were used on all variables in the models to test the hypothesis that the populations have the same distribution. We rejected the null hypothesis if the *p* value was less than 0.05.

RESULTS

Of the 379 patients enrolled (Table 1), we obtained cytokine and chemokine measurements at the initial enrollment for 211 patients (54 alcohol and 157 nonalcohol) with some individual samples being excluded due to unmeasurable levels in individual assays (141 excluded from 32-plex and 70 excluded from 35-plex). Two hundred eighty-two (74.4%) patients were men, and 143 (37.7%) were White. In univariate comparison of the alcohol and nonalcohol groups, there were two differences noted: all six Asian patients were in the nonalcohol group ($p = 0.014$) and there were statistically more men in the alcohol group than the nonalcohol group ($p = 0.015$). All other comparisons were not statistically significant ($p > 0.10$). The standardized mean difference between the two cohorts (effect size) was 0.44. Of the 211 observations, 70 (23 alcohol and 47 nonalcohol) were from the 32-plex kit and 141 (31 alcohol and 110 nonalcohol) were from the 35-plex kit (Supplementary Material).

Median illness severity score for all patients was 20, with a score of 15 or greater considered to meet major trauma criteria.[15] Patients had a median intensive care unit length of stay (LOS) of 5.8 days and hospital LOS of 12 days.

Table 2 shows the variables that were first degree associates for each MDL, and, therefore, what variables were used in final modeling at each MDL. For the 32-plex kit, there were 13 variables that were selected at all 4 MDLs. For the 35-plex kit, there were 12 variables that were selected at all 4 MDLs. For the combined set, all eight variables were selected at all four MDLs.

TABLE 1 Sc2i trauma cohort baseline characteristics of enrolled patients

Sc2i trauma cohort baseline characteristics of enrolled patients	*N* (%)
Total	379
Positive BAL	100 (26.4%)
Median age, years	35 (IQR 25–52)
Sex	
Male	282 (74.4%)
Race	
African American	217 (57.2%)
White	143 (37.7%)
Asian	6 (1.6%)
American Indian	1 (0.2%)
Not specified	12 (3.2%)
Median ISS	20
Mechanism of trauma (% penetrating)	164 (43.3%)

Abbreviations: BAL, blood alcohol level; IQR, interquartile range; ISS, illness severity score.

We determined ROC curves for both alcohol and nonalcohol models to differentiate between the alcohol cohort or the nonalcohol cohort's data. The resulting AUC were moderately successful at differentiating the cohorts, with the most successful models being the 32-plex kit at MDL 0.10 (alcohol patient set) and MDL 0.01 (nonalcohol patient set), as seen in Figure 1.

When comparing the alcohol model with the nonalcohol model graphically, we noted that the two models shared joint probability distributions (JPDs) but that there were also several unique JPDs between the two models (Figure 2). This was done to discover new systemic immune response differences in traumatically injured patients with positive BAC and those with negative BAC. IL-12 (p value = 0.01) and IP-10 (p value = 0.02) appear to be significantly different in patients with alcohol in their blood and patients without. Using connectivity, which refers to how many other nodes a variable is connected to, and model effect size indicated by a node score given by FasterAnalytics version 7.0, we found that prominent nodes in both models were IL-12, IL-6, and MCP1. We also found that in the alcohol population, MIP1A was an important node as it was more centrally connected when compared with other variables in the graph. In the nonalcohol population, IL-10, which has a large node score, compared with other variables, IL-8 and GCSF, which are centrally connected and have large node scores, appear to play large roles as well (Table 3). Our study population had a median IL-10 value of 73.5 pg/ml, with an interquartile range of 32.4–249.1 pg/ml, which far exceeds the normal range in healthy subjects (4.8–9.8 pg/ml).[16] Others have found higher levels in polytrauma patients (148 ± 33 pg/ml) and in those with polytrauma and traumatic brain injury (111 ± 30 pg/ml)[17]; however, in a mild traumatic brain injury-only group, almost all values were less than 5 pg/ml.[18]

Using single variate analyses, eight different cytokines were differentially associated with the alcohol and nonalcohol models. IL-6, IL-12, and MIP1a were central variables in the alcohol model, with the largest effect seen on MCP-1, IL-12, and IL-6. The central variables in the nonalcohol

TABLE 2 Final modeling variable selection

32-Plex Luminex kit	35-Plex Luminex kit	Combined Luminex Kit
EOTAXIN	EGF	IL-2R
FGFBASIC	HGF	IL-6
GCSF	IFNG	IL12
HGF	IL-1RA	IP10
IL-1RA	IL-2R	EGF
IL-6	IL-6	IL10
IL-8	IL-8	MIP1A
IL-10	IL-10	MIP1B
IL-12	IL-13	
IP-10	MCP1	
MCP1	MIP1A	
MIP1A	RANTES	
VEGF		

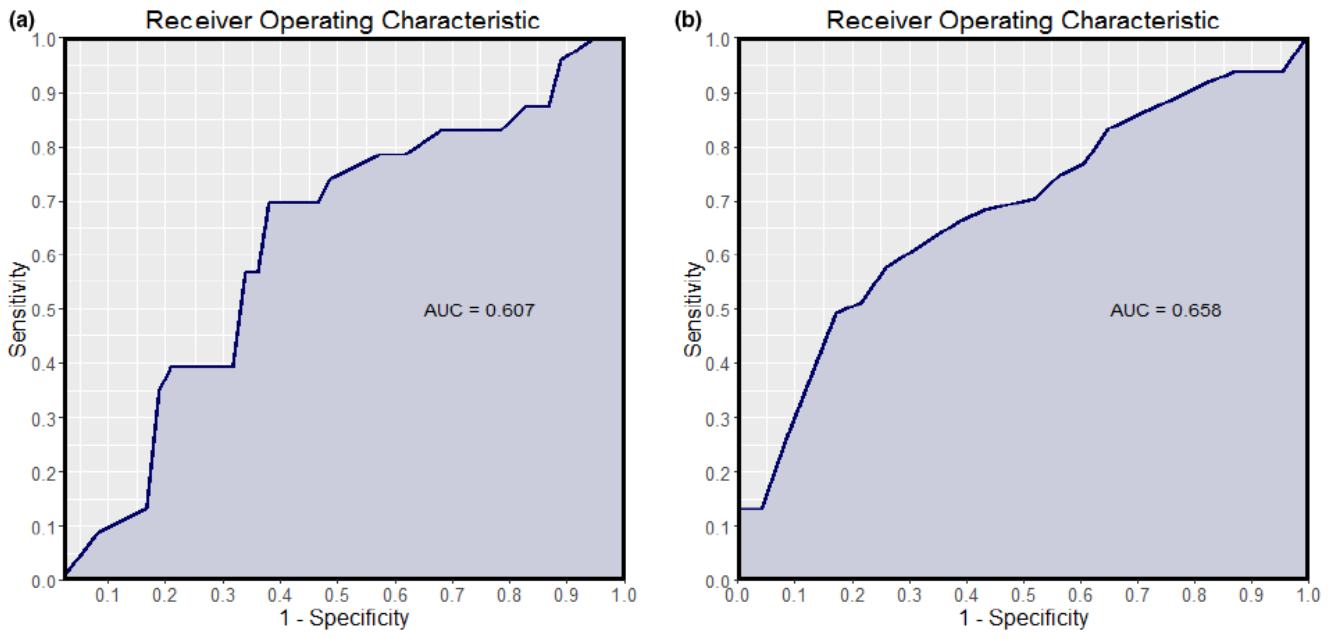

FIGURE 1 The AUC for alcohol and non-alcohol models. (a) The area under curve (AUC) for the alcohol model of the 32-plex Luminex data at minimum descriptive length (MDL) 0.10. (b) The AUC for the nonalcohol model of the 32-Plex Luminex data at MDL 0.01. The two models show modest accuracy for discriminating the cytokine values of patients with detectable serum alcohol levels at the time of their presentation versus patients without detectable serum alcohol levels

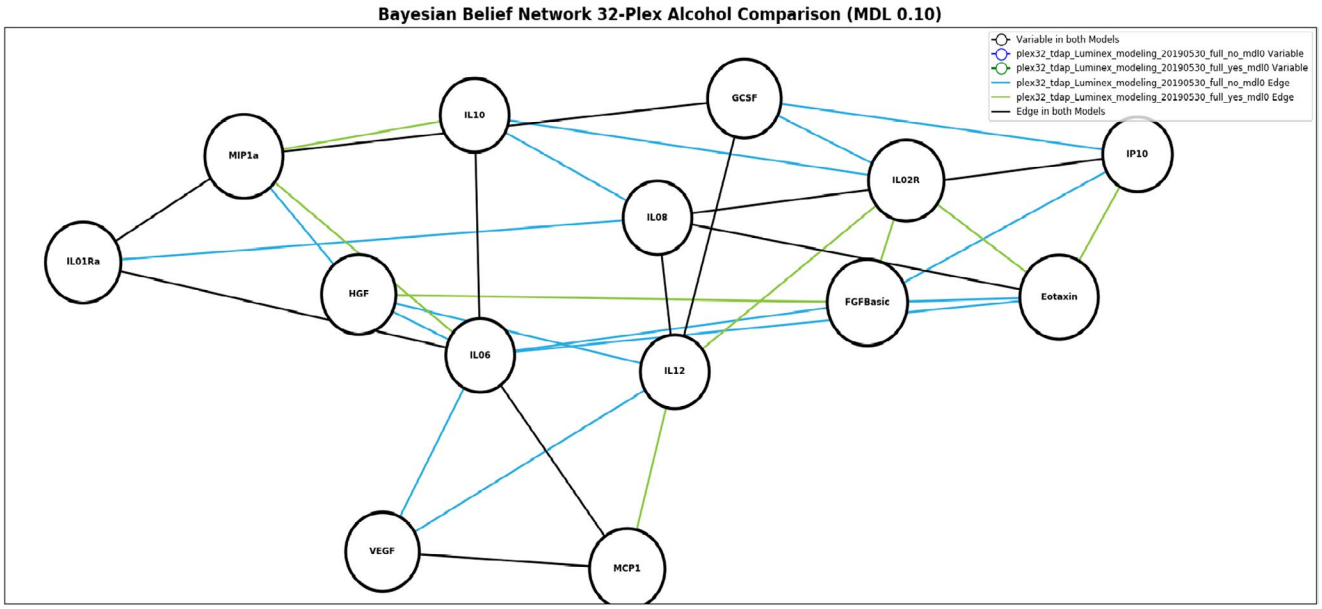

FIGURE 2 Image overlay of the 32-plex MDL in alcohol and non-alcohol models. Green lines represent the alcohol model with minimum descriptive length (MDL) of 0.10, whereas the blue lines represent the non-alcohol model with MDL of 0.01. Arcs and variables that appear in both models are in black

TABLE 3 Central and largest effect variables

Central variable	Largest effect
Alcohol model	
IL-6	MCP1
IL-12	IL-12
MIP1a	IL-6
Nonalcohol model	
IL-6	MCP1
IL-8	GCSF
IL-12	IL-8
GCSF	IL-10

model were IL-6, IL-8, I-12, and GCSF. The largest effect seen on the nonalcohol model was seen with MCP-1, GCSF, IL-8, and IL-10. Cytokines IL-12 and IL-6 were important nodes in both models and IL-10 was a prominent node in the nonalcohol model (Table 3).

DISCUSSION

The impact of alcohol on immune function in traumatically injured patients remains an important problem. In this study, we demonstrated how systematic molecular phenotyping can provide valuable insight into the possible mechanisms of morbidity in traumatically injured patients with and without alcohol in their bloodstream. By utilizing network analysis and advanced analytics, numerous variables with a relatively low sample size were able to be evaluated concurrently. Two important concepts emerged during our analysis. There was an apparent suppressing effect of alcohol on pro-inflammatory cytokine pathways involving IL-12 and IL-6, and there appeared to be greater expression of anti-inflammatory cytokine IL-10 levels in traumatically injured patients without alcohol in their blood. There still remains a need for more research, however, specifically aimed at areas of potential clinical intervention.

It is important to realize that patients unable to mount a strong immune response to traumatic injuries in the setting of positive BAC may be lacking the critically important first step of healing, inflammation. Without the assistance of cytokines signaling to initiate and regulate the immune response, it is not surprising to observe that patients may have worse outcomes due to the many complications of immune system suppression and inappropriate host defense response.

Research has established that patients with positive BAC after trauma have suppressed immune function, are more susceptible to infections, and may have decreased wound healing as compared with a patient without a positive BAC.[2] We observed the largest effects on IL-12 and IL-6 in patients with alcohol content in their bloodstream. IL-12 controls enhancement of cytotoxic activity and suppression of this cytokine decreases response to infectious or traumatic insults in alcohol-positive patients.[19] The most important function of IL-6 is to be a critical mediator of fever and the acute phase response as it is able to initiate synthesis of PGE2 in

the hypothalamus to change the body's temperature setpoint after crossing the blood–brain barrier.[20] Our findings support this prior preclinical discovery work.[21–24]

Interestingly, IL-10 appeared as a prominent variable in the nonalcohol model. The large effect on IL-10 in patients without BAC as compared with patients with positive BAC would suggest there is a relatively higher expression of this inhibitory cytokine in patients without alcohol or there is underlying mechanism suppressing IL-10 in patients with BAC. Overall, our patients had higher IL-10 levels than that found in healthy, uninjured subjects, suggesting that both mechanisms may be happening. IL-10 is known to inhibit the production of pro-inflammatory cytokines including IL-6, IL-12, and TNF-alpha, and it is thought to contribute to the potential mechanism of negative feedback regulation of the immune system.[25] This implies that overall, traumatically injured patients without BAC have a higher level of anti-inflammatory cytokine present and would therefore be better able to appropriately downregulate their immune response to trauma. This would mean they could better balance the inflammatory cascade triggered by trauma and utilize the body's own negative feedback mechanism more appropriately.[2] Conversely, if alcohol suppressed the anti-inflammatory role of IL-10, those patients might suffer from dysregulated immune response to potential infection, leading to worsened outcomes.

Utilizing network analysis of multiple variables, we have identified potentially important differences in cytokine levels in traumatically injured patients with and without BAC. There are many ways these findings could be applied clinically. In the future, it may be possible to conduct serial testing over time to understand how the variant levels of inflammatory and anti-inflammatory cytokines predict a clinical course. Developing biomarker assays that are additive to existing ones, such as complete blood count, lactate, and base deficit, for predicting severity and prognosis would be clinically valuable, and may enable clinicians to use existing resources, such as antibiotics, steroids, intensive care units, and ventilators more efficiently. Based on our analysis, assays for IL-6, IL-10, and IL-12 would be helpful. Our network analysis approach could be used to identify potential downstream effects on the cytokine pathways earlier in clinical care, for example, by identifying early on those patients who are risk for hospital-acquired infections or, conversely, identifying those who are developing acute respiratory distress syndrome from cytokine storm so that they could be treated accordingly.

System modeling provided useful data to further elucidate the relationship of alcohol on immune function in traumatically injured patients. By utilizing different patient groups and comparing them to each other with BBNs, we were able to determine cytokine interactions despite the small sample size. From this data, inferences regarding downstream effects on inflammatory and anti-inflammatory pathways can be made. We believe alcohol in the setting of traumatic injury appears to suppress cytokine pathways involved in pro-inflammatory cascade, including IL-12 and IL-6. There seems to be higher overall expression of IL-10, an anti-inflammatory cytokine, in patients without alcohol. The preliminary changes seen in IL-12, IL-10, and IL-6 need further research for possible clinical implementation.

Although our study was robust and the first of its kind examining the potential differences in traumatically injured patients with positive BAC versus patients with negative BAC, there were a few limitations. First, it should be acknowledged that cytokines' role in immune response to trauma is complex, and it is difficult to assign either pro-inflammatory or anti-inflammatory roles to individual cytokines without considering many confounding factors. It is important to note that potential confounding may have occurred as we used two different kits during the analysis period. The decision to switch to a 35-plex kit during that time was made by the laboratory staff to increase our ability to further elucidate differences in serum samples while still maintaining continuity between the studied serum samples. The differences within the kits were accounted for by analyzing both the 32-plex and 35-plex set. We therefore made significant attempts to control for any potential confounding. Second, we did not examine patient-oriented outcomes, such as infection rate or death, as we focused on the analysis of the serum samples in order to better understand the systemic differences between trauma patients with a positive BAC and trauma patients with a negative BAC. By using BBNs, we are able to quickly identify the relationships between variables likely to impact the two groups based on known probabilities. Whereas this is extremely useful for illustrating differences in the immune system of different patient groups, it does not allow us to examine patient-centered or specific outcome measures. Third, our modeling lacks external validation. Therefore, it is unclear if our models are particular to the population collected or can be reproduced in other populations. A future research goal is to collect a validation test set and use it to test these models. Bayesian modeling does not use traditional frequentist sample size calculations, but if we were to collect more data, we would look at the distribution changes to see if the population distributions were stabilizing. This would help us determine when we have collected enough data.

In conclusion, we confirmed the mechanistic hypothesis behind alcohol's impact on outcomes in traumatic injury via BBN analysis of immune cytokines in the serum of human patients. This data confirms alcohol's significant impact on the immune system seen in numerous other studies. Future directions of our study include linking our observations with patient-specific outcomes as well as examining whether the level of intoxication (BAC) correlates with the severity of the complications. Including this model in a multicenter study would be an inherently important step to allow for validation, and lead to potential treatments to

ameliorate alcohol's effect on immune function after traumatic injury by earlier identification of infection or those at risk for infection.

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

A.W.B., A.T.L., and C.A.S. wrote the manuscript. A.T.L., E.S., A.K., and E.E. designed the research. A.T.L., A.W.B., C.A., M.B.J., B.A., B.J., L.M., A.K., and E.E. performed the research. E.S. analyzed the data. M.B.J., B.A., A.K., and E.E. contributed new reagents/analytical tools.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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